Supplemental Material:

ROLE OF INTERLEUKIN 17A IN INFLAMMATION, ATHEROSCLEROSIS AND VASCULAR FUNCTION IN APOLIPOPROTEIN E-DEFICIENT MICE

Short title: Madhur *IL17A and Atherosclerosis*

ONLINE SUPPLEMENT

Meena S. Madhur[1], Samuel A. Funt[1], Li Li[1], Antony Vinh[1], Wei Chen[3], Heinrich E. Lob[1], Yoichiro Iwakura[2], Yelena Blinder[1], Ayaz Rahman[1], Arshed A. Quyyumi[1], and David G. Harrison[3]

[1]Division of Cardiology, Emory University, Atlanta, GA, USA
[2]Center for Experimental Medicine and Systems Biology, Institute of Medical Science, University of Tokyo, Tokyo, Japan
[3]Division of Clinical Pharmacology, Departments of Medicine and Pharmacology, Vanderbilt University, Nashville, TN, USA

Corresponding Author:

David G. Harrison, MD, FACC, FAHA
Professor of Medicine and Pharmacology
Director, Division of Clinical Pharmacology
Director of the Center for Vascular Biology
23rd Ave S @ Pierce Ave, 536 RRB
Vanderbilt University School of Medicine
Nashville, TN 37232-6602
Phone: (615) 875-3049
Fax: (615) 875-3297

ONLINE SUPPLEMENTAL MATERIALS AND METHODS

Animals and induction of atherosclerosis: The Institutional Animal Care and Use Committee at Emory University approved all animal protocols. IL17A$^{-/-}$ mice were generated as described in Nakae et al[1] and back-crossed to the C57BL/6J background. ApoE$^{-/-}$ mice also on a C57BL/6J background were obtained from Jackson Laboratories and crossed to IL17A$^{-/-}$ mice to generate homozygous IL17/ApoE$^{-/-}$ mice. At 8-11 weeks of age, male mice were started on a high fat diet (Purified Diet to Match Paigen's Atherogenic Rodent Diet; OpenSource Diets; Cat no D12336; 35 kcal% fat, 1.25% cholesterol, and 0.5% cholate) for 12 weeks or underwent implantation of osmotic minipumps (Alzet Model 2004, Alzet Corp) for infusion of angiotensin II (Sigma A2900) at a dose of 1000 ng/kg/min for 4 weeks. For implantation of osmotic minipumps, mice were anesthetized with ketamine (100mg/kg) and xylazine (10mg/kg) via IP injection. Minipumps were inserted after sterile preparation via a 1 cm incision between the scapula which was subsequently closed with surgical clips. Mice were sacrificed at the conclusion of experiments using CO_2 inhalation. Body weight was determined prior to start of high fat diet and at the time of sacrifice (after 12 weeks of high fat diet). Partial ligation of left carotid artery (LCA) was carried out at 8 weeks of age as previously described[2] to create low and oscillatory shear stress in the LCA. The right carotid artery (RCA) was not ligated and used as a control. ApoE$^{-/-}$ and IL17/ApoE$^{-/-}$ mice were fed the high fat diet described above for 2 weeks starting at the time of carotid ligation. For immunological clearance of IL-17F, mice were treated with anti-IL-17F neutralization polyclonal antibody (100 µg/mouse/injection, Catalog # AF2057; R&D Systems, Minneapolis, MN) or the isotype control (Goat IgG, 100 µg/mouse/injection, Catalog # AB-108-C; R&D Systems) once a week for 3 weeks starting one week before carotid ligation.

T cell isolation and Cytokine Analyses: Spleens were removed and disrupted using forceps to yield single-cell suspensions that were passed through 70 μm sterile filters. RBC lysis buffer (eBioscience) was used to remove red blood cells. Splenic T cells were then isolated using autoMACS and a T cell negative selection kit (both from Miltenyi Biotech) and cultured at a density of $2x10^5$ cells per well in 96-well anti-CD3 antibody-coated plates (BD PharMingen) for 48 hours in the presence of IL2 (R&D Systems, 20 U/ml). Cell culture supernatants were used for IL17A ELISA (Invitrogen), IL17F ELISA (R&D Systems) and Cytometric Bead Array (BD Pharmingen) studies according to the manufacturer's recommendations. Cytometric Bead Array analysis allowed for simultaneous determination of TNFα, IFNγ, IL2, IL4, and IL5 concentrations. Samples were analyzed on a LSR-II flow cytometer with DIVA software (Becton Dickinson).

Plasma Lipid Analysis: Blood was collected by cardiac puncture and centrifuged. Lipid analysis was performed commercially (Cardiovascular Specialty Laboratories, Atlanta, GA). All lipid determinations were performed using a Beckman CX7 chemistry analyzer and reagents from Beckman Diagnostics (Fullerton, CA) for total cholesterol and triglycerides.

Measurement of vascular superoxide and nitric oxide production: Superoxide production by 2 mm aortic segments was measured by quantifying formation of 2-hydroxyethidium from dihydroethidium by high-performance liquid chromatography (HPLC). This product specifically reflects the reaction of superoxide with dihydroethidium as previously validated[3]. Calcium ionophore stimulated nitric oxide production by 2 mm aortic segments was measured by electron spin resonance using the colloid probe Fe^{2+} diethyldithiocarbamate ($Fe[DETC]_2$) as previously described[4].

Aortic atherosclerotic plaque and aneurysm analyses: After sacrifice the left ventricle was punctured and the mice were pressure perfused with 0.9% sodium chloride solution followed by pressure fixation with 10% formaldehyde solution. Hearts and aortas were stored in 10% formaldehyde until analysis. The extent of atherosclerotic lesion formation in the thoracic and abdominal aorta was analyzed as follows. The aorta was dissected free of perivascular fat, opened longitudinally, and pinned down at the edges. Areas of plaque were clearly visualized as raised white lesions against the black background of the plate. Digital photographs of the aortas were used to perform planimetry using the software package ImageJ. Lesion area was expressed as percentage of aorta. To analyze plaque area in the aortic root, the hearts and part of the ascending aorta were embedded in paraffin and serial 5μm thick sections containing the aortic valve were obtained. Sections were then stained with hematoxylin and eosin and plaque area was quantified using ImageJ. Data are expressed as square microns. To analyze plaque composition, Russell-Movat pentachrome staining (American MasterTech Scientific, Inc; Item No KTRMP) was performed on paraffin embedded sections of the aortic root according to the manufacturer's protocol.

Aneurysm incidence (defined as greater than 50% dilation of the aorta) was recorded upon sacrifice and dissection of mice that were infused for 4 weeks with angiotensin II. Occasionally, mice died during angiotensin II infusion. In these cases, autopsy was performed to detect the presence of an abdominal aneurysm or a retroperitoneal hematoma, suggesting rupture of an abdominal aneurysm.

Carotid atherosclerosis analysis and Oil red O staining: Mice were sacrificed and perfused at physiological pressure with saline containing heparin. The left and right carotid arteries were removed en bloc with the trachea and esophagus. For frozen sections, tissue was embedded in Tissue-Tek optimum cutting temperature medium, frozen in liquid nitrogen, and stored at -80°C until stained. Oil red O staining was carried out using frozen sections as previously described[2] and nuclei were stained with hematoxylin. Images were captured with a Zeiss epifluorescence microscope. Images were analyzed with Image J software to quantify lesion size as previously described[2].

Immunohistochemistry for macrophages: Seven μm frozen sections of the aortic root were prepared. Immunostaining was performed using a rat anti-mouse Mac3 antibody (BD Pharmingen; Cat No 550292) at a 1:50 dilution for 1 hour at room temperature and a secondary biotinylated goat anti-rat IgG (Vector; Cat No BA 9400) at a 1:1000 dilution for 30 min at room temperature. A Vectastain alkaline phosphatase system (Vector AK-5000 and Vector SK-5100) with levamisole (Vector SP-5000) to

inhibit endogenous alkaline phosphatase activity was used to detect the secondary antibody. All slides were counterstained with hematoxylin.

Flow Cytometry: A separate set of ApoE$^{-/-}$ and IL17/ApoE$^{-/-}$ mice were sacrificed to analyze cell composition within the vessel wall using flow cytometry. Whole aortas were removed and dissected free of perivascular fat. Harvested aortas were digested using collagenase type IX (125u/ml); collagenase type IS (450U/ml) and hyaluronidase IS (60U/ml) dissolved in 20 mM HEPES-PBS buffer containing calcium and magnesium for 30 minutes at 37^{o} C, while constantly agitated. The dissolved tissue was then passed through a 70 μm sterile filter (Falcon, BD), yielding a single cell suspension. Cells were washed twice with PBS buffer and then stained with a live/dead cell stain (near infrared, Invitrogen). After washing with 1% BSA PBS buffer, cells were blocked for nonspecific Fc binding with anti-mouse CD16/32 (2.4G2) and then stained using the following fluorescent antibodies: FITC-CD45 (total leukocytes, 30-F11), PE-CD3 (T cells, 145-2C11), PerCP-Cy5.5-CD11c (HL3), APC-CD11b (M1/70) and PE-Cy7-F4/80 (macrophages, BM8). Antibodies were purchased from BD Biosciences except for PE-Cy7-F4/80 (eBioscience).

Flow cytometry was performed using a LSR-II flow cytometer with DIVA software (Becton Dickinson). Countbright counting beads (Invitrogen) were used to normalize total cell counts among samples. At least 10,000 bead events were acquired to insure the accuracy of the assay. Absolute cell count (cells/aorta) = events of cells counted/total number of beads counted x input bead number. CD11b^{+}F4/80^{+} cells were defined as macrophages; and CD11b^{-}CD11c^{+} cells were defined as dendritic cells. Gating was applied using fluorescence minus one (FMO) controls constructed with staining panels where one of the fluorescent markers of interest was replaced with an isotype control. Data were analyzed with Flowjo software 9.1 (Treestar).

Patient population, analysis of serum IL17A levels, and carotid intima-media thickness (IMT) determinations: Sixteen study subjects for whom serum samples and carotid IMT data were available were chosen at random from a pre-existing database at Emory University Hospital. Institutional Review Board approval was obtained for the human studies. Of these subjects, 10 were male and 6 were female. The age range was 50-69 years with a mean age of 58 years. The average BMI was 26, average systolic blood pressure 117 mmHg, and average LDL 114 mg/dL.

The Fluorokine® MultiAnalyte Profiling (MAP) Human base Kit A (Catalog #: LUH000) from R&D Systems (Minneapolis, USA) was used to determine the levels of IL17 analyte (Catalog # LUH317) on a Luminex 100 Bio-Plex platform (Bio-Rad, CA USA). All samples are centrifuged before testing and all assays are performed as directed by manufacturer.

Carotid IMT, the distance between the junction of the lumen and intima and that of the media and adventitia, was measured by means of B-mode ultrasound of the carotid arteries. IMT was quantified both on the near and far wall at the distal 1.0 cm of the left and right common carotid arteries proximal to the bifurcation and the bifurcation itself, therefore yielding a total of 8 segments (4 on each side). For each segment, the sonographer used 5 different scanning angles to identify the optimal longitudinal image of IMT (showing the greatest IMT), and 3 images of the latter were frozen and stored on

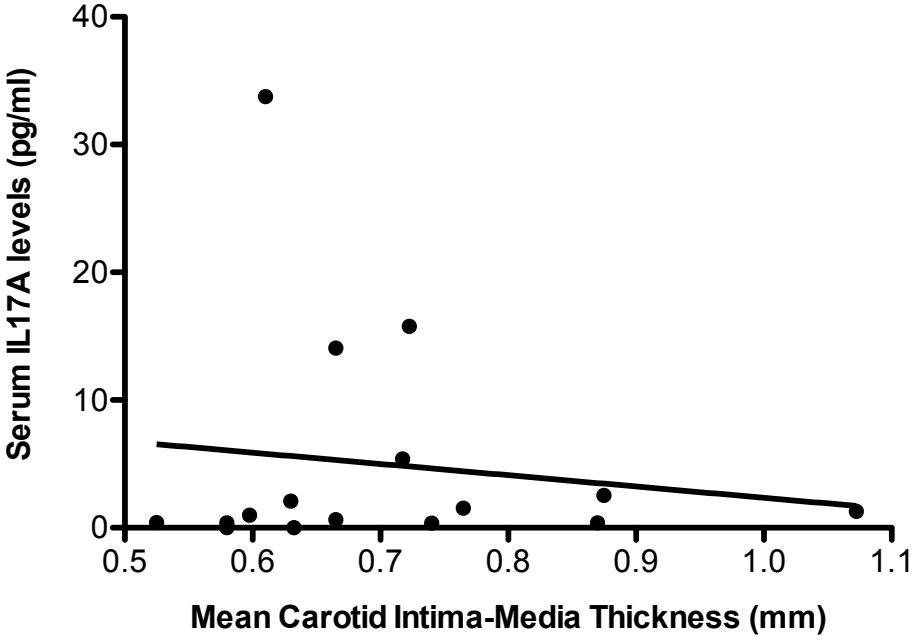
optic disk. The actual measurements of IMT were performed off-line using a semi-automated computerized analytical software (Carotid tools, MIA Inc., Iowa City, USA), by 2 observers blinded to the test results. The IMT of each of the 3 frozen images for each segment was averaged to determine the mean IMT for that segment at the near and far wall, at each side and both sides combined.

Statistics: Data are expressed as mean $\pm$ standard error of the mean. When comparing only 2 groups, Student's t-test was used. For comparisons involving 3 or more groups, one-way ANOVA followed by Neuman-Keuls post-hoc test was employed. For body weight analysis prior to and after high fat diet, we employed ANOVA for repeated measures. For the interleukin 17F ELISA analysis, Student's t-test was employed with a Bonferroni correction for 4 comparisons. The p values provided reflect the Bonferroni correction. A p value ≤ 0.05 was considered significant.

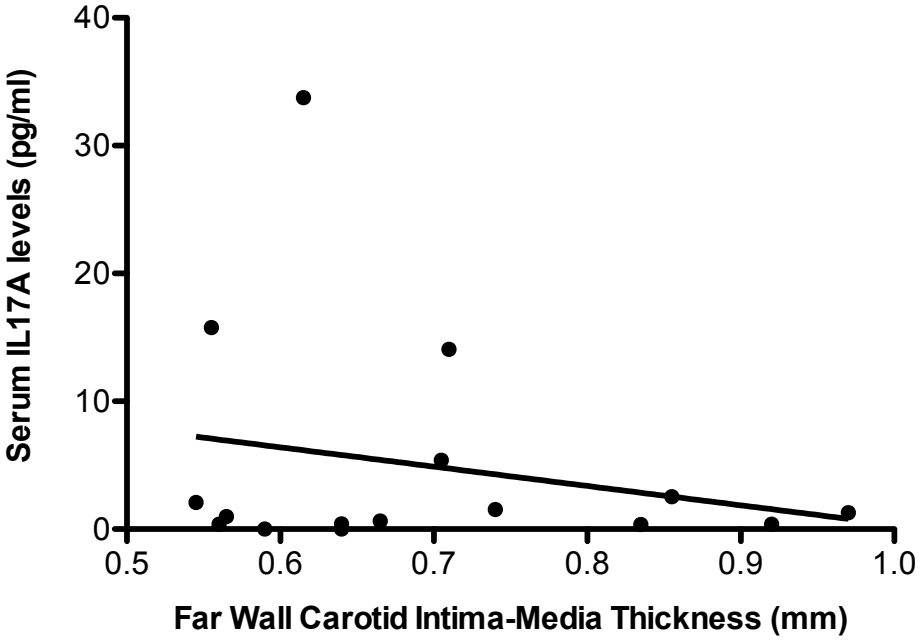
REFERENCES FOR ONLINE SUPPLEMENTAL MATERIALS AND METHODS

1. Nakae S, Komiyama Y, Nambu A, Sudo K, Iwase M, Homma I, Sekikawa K, Asano M, Iwakura Y. Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses. *Immunity.* 2002;17:375-387.
2. Nam D, Ni CW, Rezvan A, Suo J, Budzyn K, Llanos A, Harrison D, Giddens D, Jo H. Partial carotid ligation is a model of acutely induced disturbed flow, leading to rapid endothelial dysfunction and atherosclerosis. *Am J Physiol Heart Circ Physiol.* 2009;297:H1535-1543.
3. Fink B, Laude K, McCann L, Doughan A, Harrison DG, Dikalov S. Detection of intracellular superoxide formation in endothelial cells and intact tissues using dihydroethidium and an HPLC-based assay. *Am J Physiol Cell Physiol.* 2004;287:C895-902.
4. Dikalov S, Fink B. ESR techniques for the detection of nitric oxide in vivo and in tissues. *Methods Enzymol.* 2005;396:597-610.

A


Serum IL17A levels (pg/ml)
40
30
20
10
0
0.5
0.6
0.7
0.8
0.9
1.0
1.1
Mean Carotid Intima-Media Thickness (mm)

B


Serum IL17A levels (pg/ml)
40
30
20
10
0
0.5
0.6
0.7
0.8
0.9
1.0
Far Wall Carotid Intima-Media Thickness (mm)

Supplemental Figure: Correlation of Serum IL17A levels with Carotid Intima-Media Thickness in Humans. No correlation was observed between mean (A) and far wall (B) carotid intima-media thickness and serum IL17A levels in 16 healthy humans aged 50-69 years (p=n.s.).